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Supporting Information for

Engineering Metal-Based Hydrogel Mediated Tertiary Lymphoid Structure Formation via Activating STING Pathway for Enhanced Immunotherapy

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MATERIALS AND METHODS

Materials. $Zn(NO_3)_2 \cdot 6H_2O$ was purchased from Sinopharm Chemical Reagent Co., Ltd.. 4,5-imidazoledicarboxylic acid (IDA) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd.. Chitosan and sodium tripolyphosphate was purchased from Shanghai Macklin Biochemical Technology Co., Ltd. CpG was purchased from Sangon Biotech (Shanghai) Co., Ltd.. MitoSOX Red Mitochondrial Superoxide Indicator was purchased from ABclonal Technology Co., Ltd.. Enzyme linked immunosorbent assay (ELISA) kit for IFN-β, INF-γ, TNF-α, IL-6 and IL12p70 was purchased from 4A Biotech Co., Ltd (Beijing, China). Recombinant murine IL-4, recombinant murine GM-CSF and methyl thiazolyl tetrazolium (MTT) were purchased from Beyotime Biotech. Inc.. Roswell Park Memorial Institute (RPMI) 1640 culture medium, Gibico®Opti-MEM medium and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from Biological Industries.

Synthesis and characterization of chitosan nanoparticles (CSNPs). CSNPs were prepared using an ionotropic gelation method. Firstly, chitosan (0.3 g) was dissolved in 1% v/v acetic acid to obtain a 1.5 mg/mL CS solution. The solution was stirred at 50 ℃ and the pH was adjusted to 5 ~ 6 by NaOH (1 M). Then, 7.5 mL sodium tripolyphosphate (TPP, 0.8 mg/mL) was added dropwise to 20 mL chitosan solution, and the solution was further stirred for 3 h to prepare chitosan nanoparticles. The morphology of CSNPs was observed by TEM (JEM-2100 Plus) and SEM (Zeiss Sigma). The DLS size distribution and zeta potential of CSNPs was measured by using Zetasizer Nano ZS (Malvern Instruments).

Synthesis and characterization of ZIF and Zn-Gel (ZG). In brief, $Zn(NO_3)_2 \cdot 6H_2O$ (200 mg) was firstly dissolved in methanol (10 mL). 4,5-Imidazoledicarboxylic acid (IDA, 400 mg) was dissolved in the solution containing methanol (10 mL) and 5 mL Tris-HCl buffer (pH = 8.0). The zinc nitrate–methanol solution was mixed with IDA and further stirred for 2 h. The synthesized ZIF nanoparticles were centrifuged at 4500 rpm and washed with methanol for three times. Finally, the resulting precipitate was dispersed in water to obtain a transparent ZG. To prepare the final hydrogel (ZCCG), predetermined amounts of CSNPs and CpG were mixed with the precursor hydrogel. The hydrogels containing CSNPs or CpG were named ZCsG and ZCpG, respectively. The morphology of ZIF was detected using TEM and SEM, and its size distribution and zeta potential were measured by Zetasizer. The gelation process was supervised via a rotational rheometer r (TA-Waters, DHR-2) at 37 ℃, and the morphology of ZG and ZCCG was observed using SEM.

pH-responsive release of Zn^{2+} from ZCCG. The Zn^{2+} release of ZCCG in PBS under different pH (5.4, 6.5 and 7.4) was investigated. This was conducted by adding 1 mL ZCCG into 20 mL PBS at 37 ℃. At a series of points in time, the supernatant was collected and detected by ICP-MS (Analytik Jena, PQ-MS) to

determine the Zn^{2+} concentration.

Cell experiments. Murine 3T3 cells, 4T1 cells, B16-F10 cells and DC 2.4 cells were purchased from China Center for Type Culture Collection (CCTCC) and cultured in RPMI 1640 or DMEM containing 10% FBS and penicillin-streptomycin at 37 °C with 5% CO_2. Bone marrow dendritic cells (BMDCs) were isolated from the C57BL/6J mice. Briefly, the bone mesenchymal stem cells were gained from the bone marrow cavities of mice femurs, and then they were cultured in RPMI 1640 culture medium containing IL-4 and GM-CSF for 7 days, and the non-adherent cells were harvested on day 8 for further experiments.

Cell viability assay. Murine 3T3 cells or 4T1 tumor cells were used to evaluate cytocompatibility of hydrogels by MTT in vitro. All cells (1×10^4 cells/well) were seeded in 96-well plates for 12 h. Cells were incubated with PBS, ZG, ZCsG, ZCpG, and ZCCG for 48 h. MTT was added to these wells after removing the medium containing hydrogels and incubated for 4 h. Then, the formazan was dissolved by DMSO. The absorbance at 490 nm was measured.

Cellular uptake analysis. The cellular internalization mechanism of CSNPs and CpG was explored. Briefly, DC2.4 cells (1×10^5 cells/well) were seeded in 6-well plates and incubated for 24 h. Subsequently, cells were treated with PBS, methyl-β-cyclodextrin (2 mM), sucrose (450 mM), amiloride (2 mM) or 4 ℃, respectively, and were cultured for 1 h. Then, ZCCG (FAM-labeled CSNPs or FAM labeled CpG) was added into the medium in weak acid (pH = 6.5). After cultured for 8 h, the cells were washed with PBS, and detected by flow cytometry.

DC2.4 cells (1×10^5 cells/well) were seeded in 6-well plates and incubated for 24 h. After that, PBS and ZCCG (FAM-labeled CSNPs or FAM labeled CpG) were incubated with DC2.4 cells in the medium in weak acid (pH = 6.5) for 8 h. Then, cells were staining with LysoTracker™ red and analyzed via the fluorescence microscope. For the colocalization of CSNPs and mitochondria, the cells were treated with PBS and ZCCG (FAM-labeled CSNPs) in the medium in weak acid (pH = 6.5) for 12 h. Then, cells were staining with MitoTracker™ deep red and analyzed via the fluorescence microscope.

cGAS-STING pathway activation evaluation in vitro. To measure the intracellular mitochondrial ROS level, DC 2.4 cells were seeded in 6-well plates, and cultured in the medium in weak acid (pH = 6.5)

overnight. Then, different hydrogels were added. After another 48 h, the cells were washed with PBS and stained with MitoSOX. The fluorescence images and intensity were evaluated by fluorescence microscopy and flow cytometry, respectively. Then, to evaluate intracellular oxidized mitochondrial DNA caused by the hydrogel, DC 2.4 cells were seeded on coverslip and cultured overnight. Then, different hydrogels were added. After another 48 h, the cells were washed with PBS and stained with 8-OHdG antibody and TOMM20 antibody to measure oxidized mitochondrial DNA. Likewise, the intracellular mitochondrial ROS level and oxidized mitochondrial DNA were also measured in 4T1 tumor cells in the same way. To measure the intracellular cGAMP levels, DC 2.4 cells were seeded in 6-well plates, and cultured in the medium in weak acid (pH = 6.5) overnight. Then, different hydrogels were added. The cells were collected and detected with a cGAMP ELISA kit. Furthermore, BMDCs were used to evaluate the ability of hydrogels for promoting DC maturation in vitro. BMDCs were seeded in 6-well plates with the medium in weak acid (pH = 6.5) and co-cultured with PBS, ZG, ZCsG, ZCpG or ZCCG for 48 h. Then, the cells were stained with anti-CD11c-FITC, anti-CD86-APC and anti-CD80-PE antibodies. The DCs maturation was detected using flow cytometry (BD AccuriTM C6). Western blot (WB) was used for the detection of STING pathway activation in vitro. DC 2.4 cells were seeded in 6-well plate overnight, and the cells were treated with PBS, ZG, ZCsG, ZCpG or ZCCG. After 48 h incubation, the STING pathway- and TLR9- related protein expression was measured by western blot. To measure the T cells activation *in vitro*, the BMDCs after various treatments were co-cultured with T cells for 24 h. Then, the medium was collected for cytokines detection (TNF-α and IFN-γ) via ELISA kits. Furthermore, these T cells were co-cultured with 4T1 cells, and the medium was collected for the detection of LDH levels.

Animals and Tumor Models. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the Animal Experiment Center of Wuhan University (Wuhan, China). All experimental procedures were performed following the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China. Subcutaneous B16 or 4T1 tumor models were established with female C57BL/6 mice (6–8 weeks) and Balb/c mice (6–8 weeks), respectively.

***In vivo* antitumor immunity.** 3×10^5 B16-F10 cells were subcutaneously injected into the right flank of C57BL/6 mice to construct B16-F10 primary tumor models. A week later, the mice were randomly divided into five groups and intratumorally administered with PBS, ZG, ZCsG, ZCpG or ZCCG. The tumor volumes and body weight of each mouse were recorded every other day. After the administration for 3 or 8 days, the tumors and lymph nodes were obtained for the analysis of immune effects by flow cytometry. Tumors were

digested at 37 °C for 2 h to obtain a single-cell suspension. For analyzing the infiltrating lymphocytes, the cells were stained with anti-CD3-FITC, anti-CD4-APC, anti-CD8a-PE, anti-CD62L-FITC, anti-CD44-APC, anti-CD19-FITC and anti-CD20-APC antibodies. To analyze the $CD11c^+CD80^+CD86^+$ DCs, the lymph nodes in mice were collected to obtain lymphocytes, and the lymphocytes were stained with anti-CD11c-FITC, anti-CD86-APC and anti-CD80-PE antibodies. The stained cells were measured by flow cytometry. Of note, intratumoral cytokines, including IFN-β, cGAMP, CXCL10, IL-6, IL-12p70, IFN-γ, and TNF-α, were detected by ELISA kits as the manufacturer's methods.

Cytokine neutralization. 3×10^5 B16-F10 cells were subcutaneously injected into the right flank of C57BL/6 mice to construct B16-F10 primary tumor models. A week later, the mice were randomly divided into five groups and intratumorally administered with PBS, ZCCG+αIFN-γ, ZCCG+αTNF-α, ZCCG+αIFNAR-1 or ZCCG. Antibodies (100 μg per mouse) were intraperitoneal injected on days 10 and 11. The tumor volumes and body weight of each mouse were recorded every other day.

Transcriptomic analysis. 3×10^5 B16-F10 cells were subcutaneously injected into the right flank of C57BL/6 mice to construct B16-F10 primary tumor models. A week later, the mice were randomly divided into two groups and intratumorally administered with PBS or ZCCG. Meanwhile, the tumor was collected the next day. The high-throughput sequencing was conducted in Majorbio BioTech Co., Ltd.

Improvement of the immunosuppressive TME by ZCCG. 3×10^5 B16-F10 cells were subcutaneously injected into the right flank of C57BL/6 mice to construct B16-F10 primary tumor models. A week later, the mice were randomly divided into two groups and intratumorally administered with PBS or ZCCG. On days 6, 9 and 12, ZCCG was implanted into the tumors The tumor volumes and body weight of each mouse were recorded every other day. The tumors and lymph nodes of four mice in each group were obtained for the analysis of immune effects by flow cytometry on day 15 to analyze the frequencies of maturated DCs ($CD11c^+CD80^+CD86^+$), $CD8^+$ T cells ($CD3^+CD4^-CD8^+$), $CD4^+$ T cells ($CD3^+CD4^+CD8^-$), Tregs ($CD3^+CD4^+Foxp3^+$), M1 ($CD11b^+F4/80^+CD80^+$), M2 ($CD11b^+F4/80^+CD206^+$), and MDSC ($CD45^+CD11b^+Gr\text{-}1^+$).

Anti-tumor immune response of ZCCG combined with αPD-1 *in vivo*. 3×10^5 B16-F10 cells were subcutaneously injected into the right flank of C57BL/6 mice to construct B16-F10 primary tumor models. A week later, the mice were randomly divided into five groups and intratumorally administered with PBS, αPD-1, ZCCG or ZCCG+αPD-1. On days 6, 9 and 12, ZCCG was implanted into the tumors. αPD-1 was administered via intraperitoneal injection on days 7, 10 and 13. The tumor volumes and body weight of each mouse were recorded every 2 d. The tumors and lymph nodes of four mice in each group were obtained for

the analysis of immune effects by flow cytometry on day 15 to analyze the ratio of the DC cells ($CD11c^+CD80^+CD86^+$), $CD3^+CD4^+$ and $CD3^+CD8a^+$ T cells, the memory T cell ($CD8a^+CD44^+CD62L^+$ and $CD8a^+CD44^+CD62L^-$), and the B cells ($CD19^+CD20^+$) as before.

Anti-tumor ability of ZCCG combined with αPD-1 on 4T1 tumor models. 3×10^5 4T1 cells were subcutaneously injected into the right flank of BALB/c mice to construct 4T1 primary tumor models, and the mice were treated with PBS, αPD-1, ZCCG or ZCCG+αPD-1. ZCCG was implanted into the tumors on days 6, 9 and 12. αPD-1 was administered via intraperitoneal injection on days 7 and 10. The tumor volumes and body weight of each mouse were recorded every other day. Spontaneous metastases were detected by H&E staining. On day 90, the survived mice in ZCCG+αPD-1 group were subcutaneously injected 5×10^5 4T1 cells to evaluate the immunological memory effect.

ELISA. Tumor tissues were collected after various treatments. The tumor tissues were weighed after collection, homogenized and centrifuged at 12,000 rpm for 10 min for supernatant collection. Cytokines and chemokines were measured using ELISA kits according to the manufacturer's protocol.

Flow cytometry and Antibody. Tumors were digested in 1640 medium with 2% BSA containing 1 mg/mL collagenase IV (Biosharp), 1 mg/mL hyaluronidase (Yeasen Biotechnology (Shanghai) Co., Ltd), and 10 μg/mL DNase I (Yeasen Biotechnology (Shanghai) Co., Ltd) at 37 °C for 2 h to obtain a single-cell suspension, and red blood cells were removed with lysis buffer. All antibodies are from Biolegend unless otherwise noted. The following flow cytometry antibodies were used: DCs (anti-CD11c-FITC, anti-CD86-APC and anti-CD80-PE antibodies), $CD4^+$ and $CD8^+$ T cells (anti-CD3-FITC, anti-CD4-APC and anti-CD8a-PE), memory T cells (anti-CD8a-PE anti-CD62L-FITC and anti-CD44-APC), B cells (anti-CD19-FITC and anti-CD20-APC), Treg cells (anti-CD3-FITC, anti-CD4-APC and anti-Foxp3-PE), M1 macrophages (anti-CD11b-PE, anti-F4/80-APC and anti-CD86-FITC), M2 (anti-CD11b-PE, anti-F4/80-APC and CD206-FITC), and MDSC (anti-CD45-PE, anti-CD11b-APC and anti-Gr-1-FITC). Flow cytometry data were collected using BD Accuri™ C6 software and analysed using FlowJo v.10.

Western blot analysis. Cell lysates and tissue lysates were centrifuged at 12,000 rpm for 5 min at 4 °C, and the total protein was quantified via BCA assay (ASPEN). Loading buffer was added into supernatants and boiled for 5 min. Forty μg of protein was extracted from the different samples and separated in an electrophoresis chamber in 8% SDS-PAGE (Beijing Liuyi Biotechnology Co., Ltd., DYY-6C, China). Then, proteins were transferred to PVDF membranes (Millipore) and activated with methanol. The blots were then incubated with different primary antibodies against overnight at 4 °C, followed by incubation with the horseradish peroxidase (HRP)-conjugated antirabbit IgG at room temperature for 30 min. Finally, the bands

were imaged by electrochemiluminescence western-blotting substrate detection.

Statistical analysis. GraphPad Prism 8 software was used for statistical analysis. Statistical data are shown as the mean ± s.e.m. unless otherwise stated. Details of the statistical test, P values and number of replicates are provided in the figures. Significance of $P<0.05$ was used as the threshold for all tests.

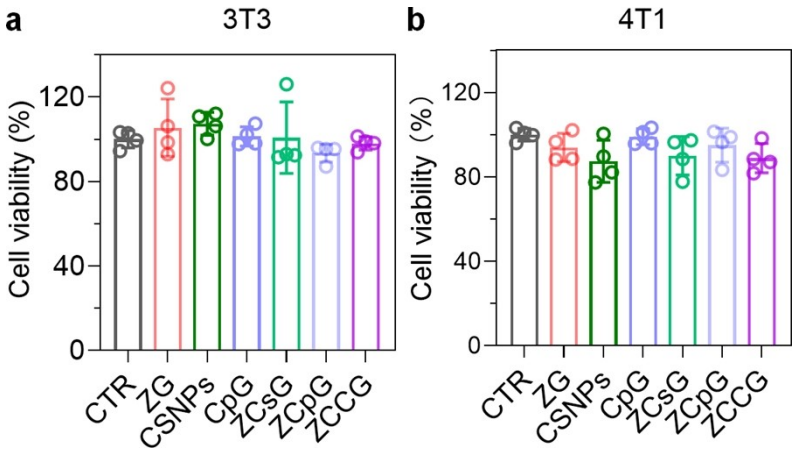

Fig. S1 a-b) Cytocompatibility of different treatments toward 3T3 cells and 4T1 cells.

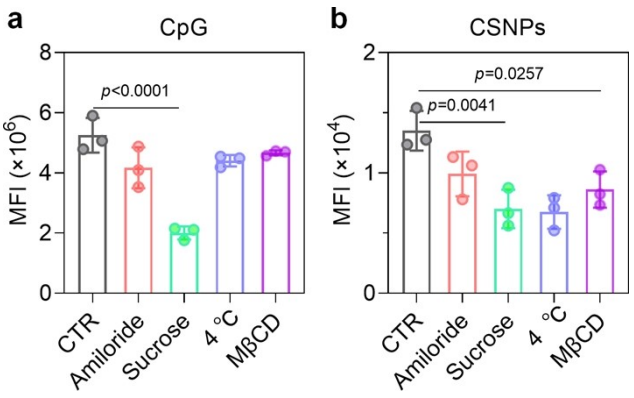

Fig. S2 a-b) Endocytosis inhibition of CpG and CSNPs under different inhibitors and low temperature.

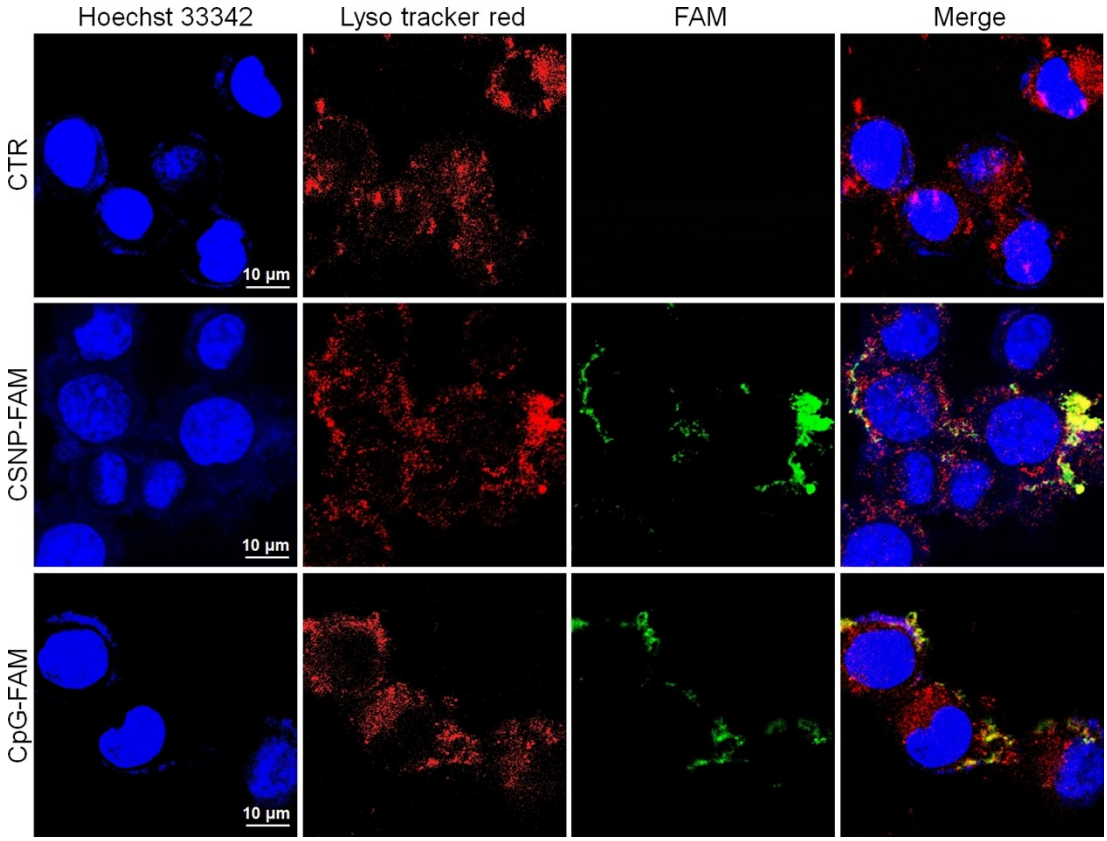

Fig. S3 Representative immunofluorescence images of intracellular CSNPs-FAM released from ZCCG (CSNPs-FAM and CpG) or CpG-FAM released from ZCCG (CSNPs and CpG-FAM) in DC2.4 cells.

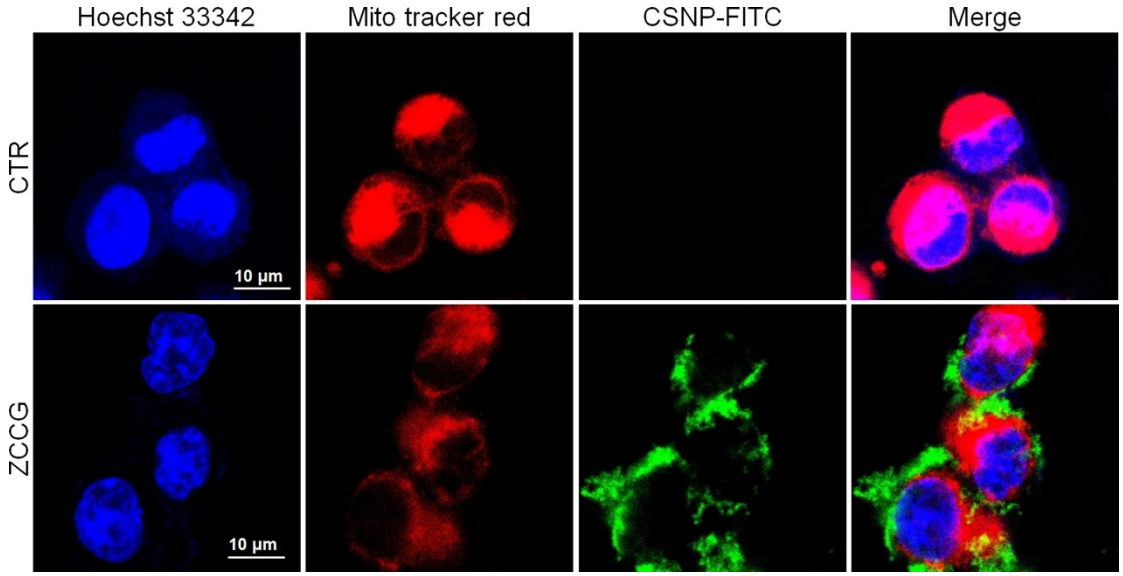

Fig. S4 Representative immunofluorescence images of the colocalization of CSNPs-FAM released from hydrogel and mitochondria of DC2.4 cells in vitro.

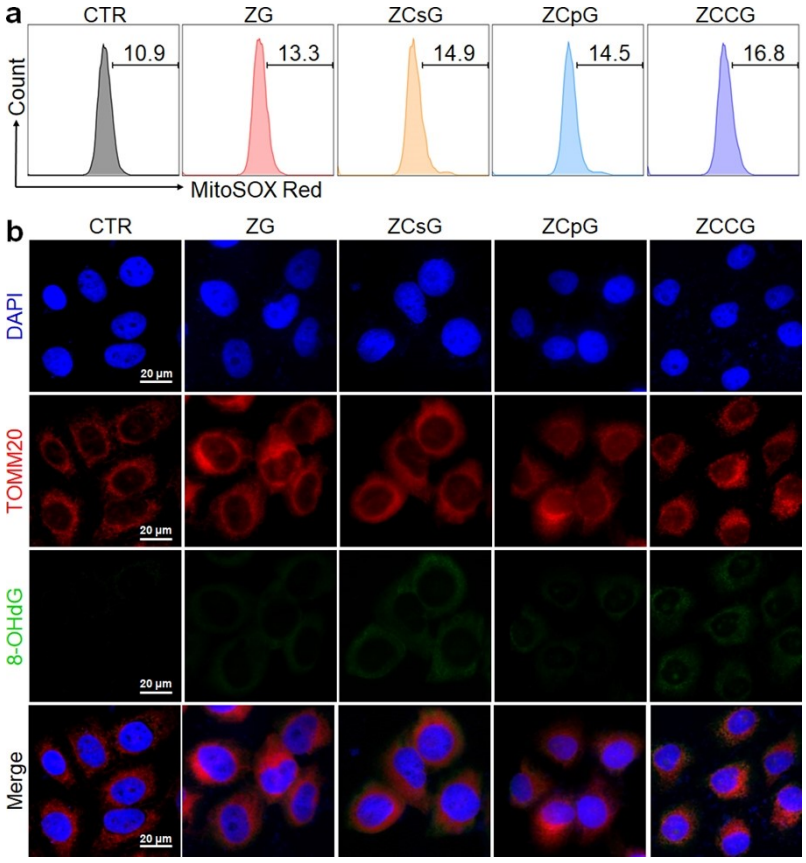

Fig. S5 a) Representative immunofluorescence images of mitoROS level in 4T1 tumor cells. **b**) Representative immunofluorescence images of mitoDNA oxidation levels inside treated 4T1 tumor cells.

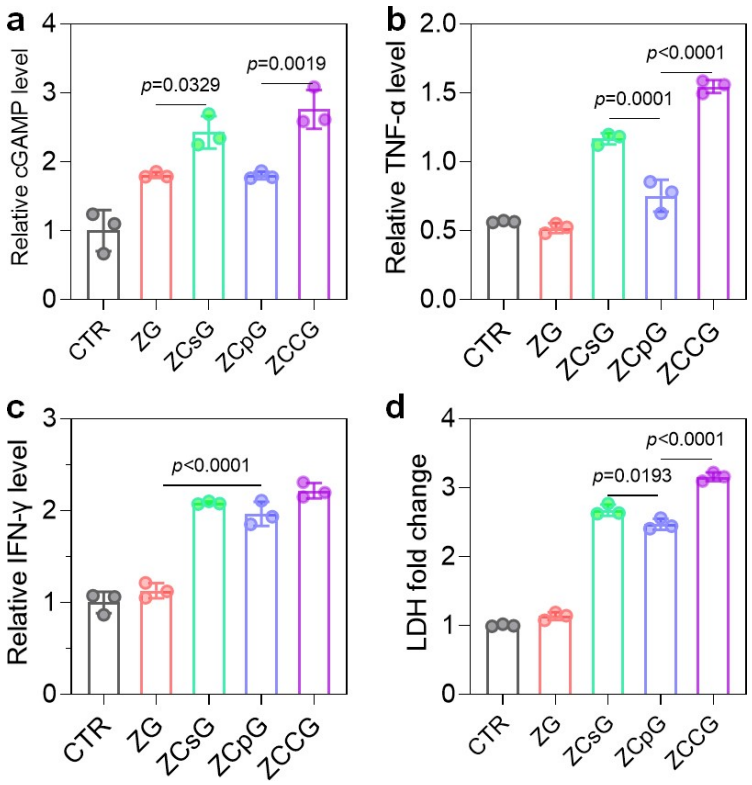

Fig. S6 a) Intracellular cGAMP levels after diverse treatments in DC2.4 cells. **b-c**) TNF-α and IFN-γ levels in the medium after the co-incubation of BMDCs and T cells. **d**) LDH levels of 4T1 tumor cells after being co-cultured with T cells.

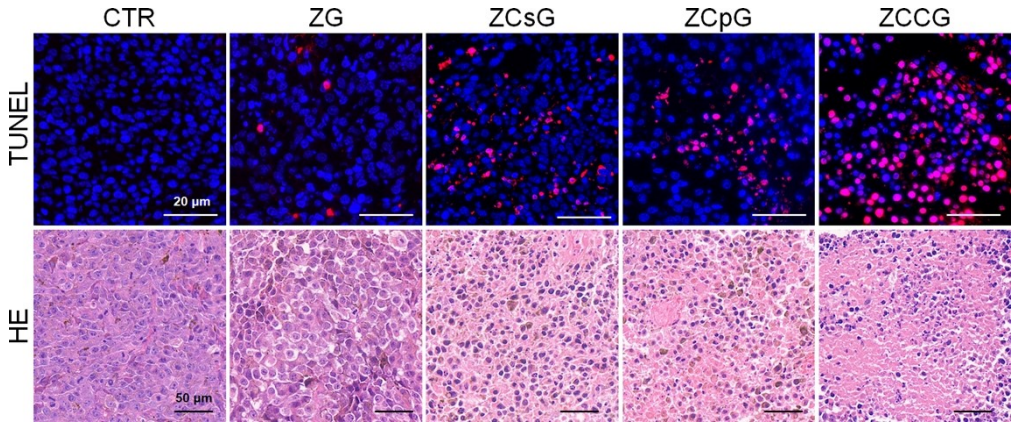

Fig. S7. TUNEL and HE staining of tumor sections after various treatments.

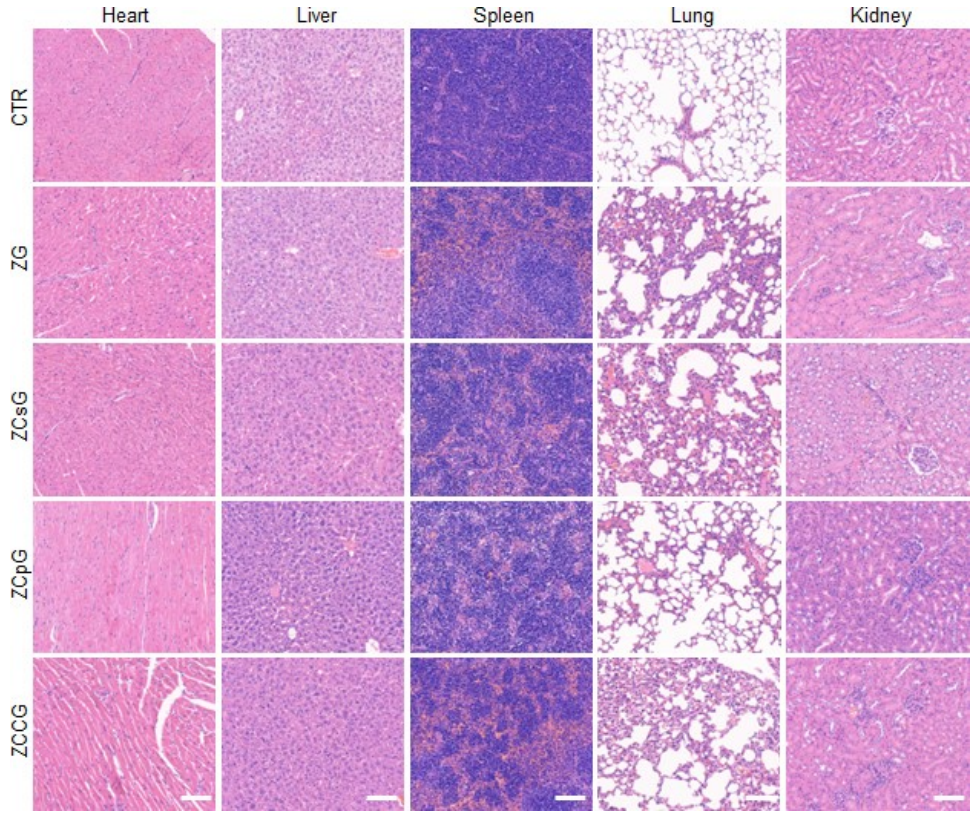

Fig. S8. H&E staining images of main organ slices from tumor bearing mice with various treatments. Scale bar: 100 μm.

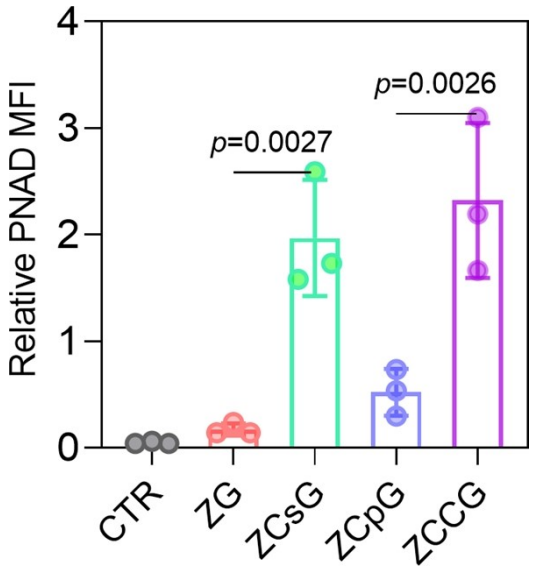

Fig. S9. The corresponding quantification of fluorescence intensity in Figure 4q using ImageJ software from four randomly selected images.

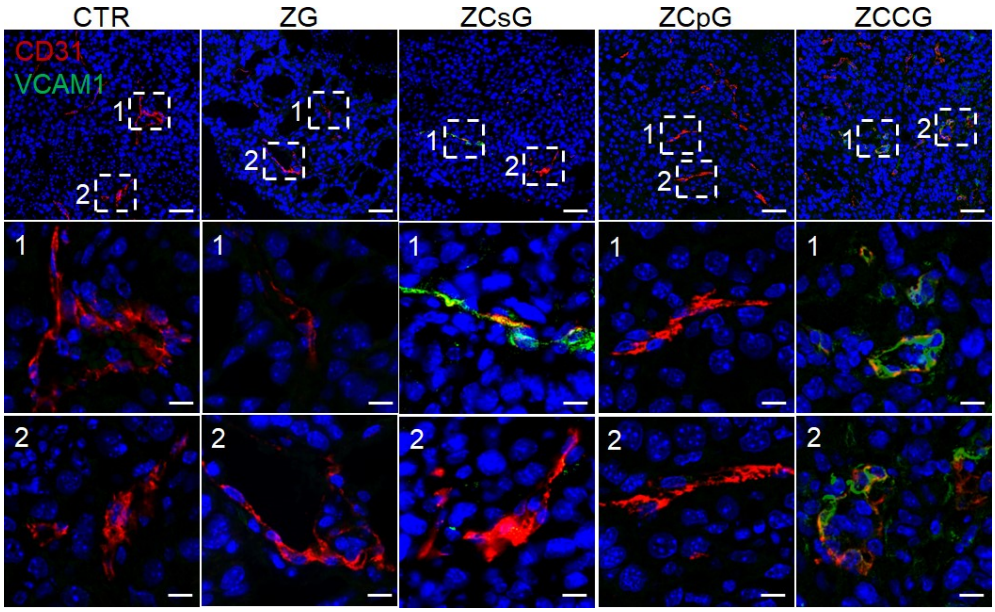

Fig. S10. Immunofluorescence images of tumor sections for CD31 (red) and VCAM1 (green). Scale bar: 50 μm.

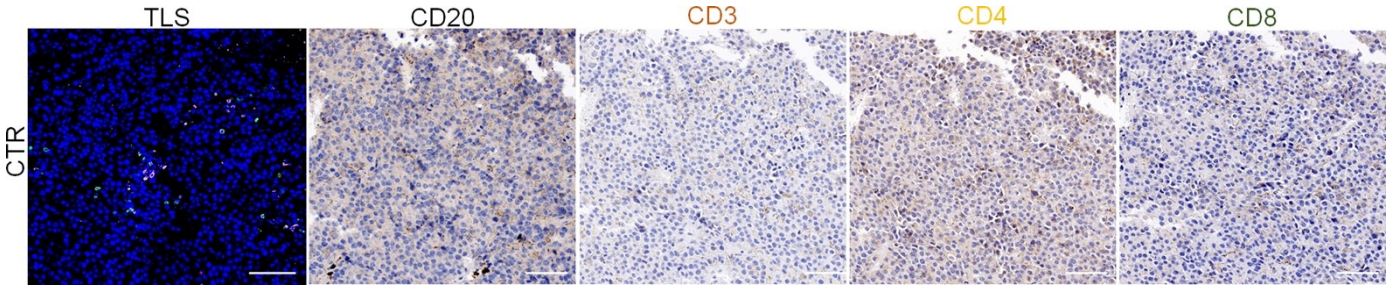

Fig. S11. Immunofluorescence and immunohistochemical images of tumor sections in the control group. Scale bar: 50 μm.

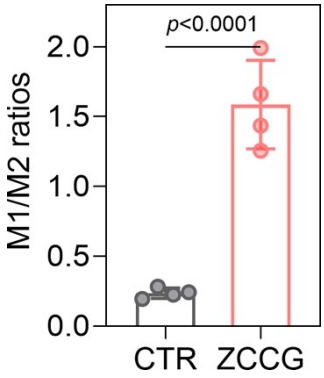

Fig. S12. The quantification results of the ratios for M1/M2 macrophages within TME after different treatments.

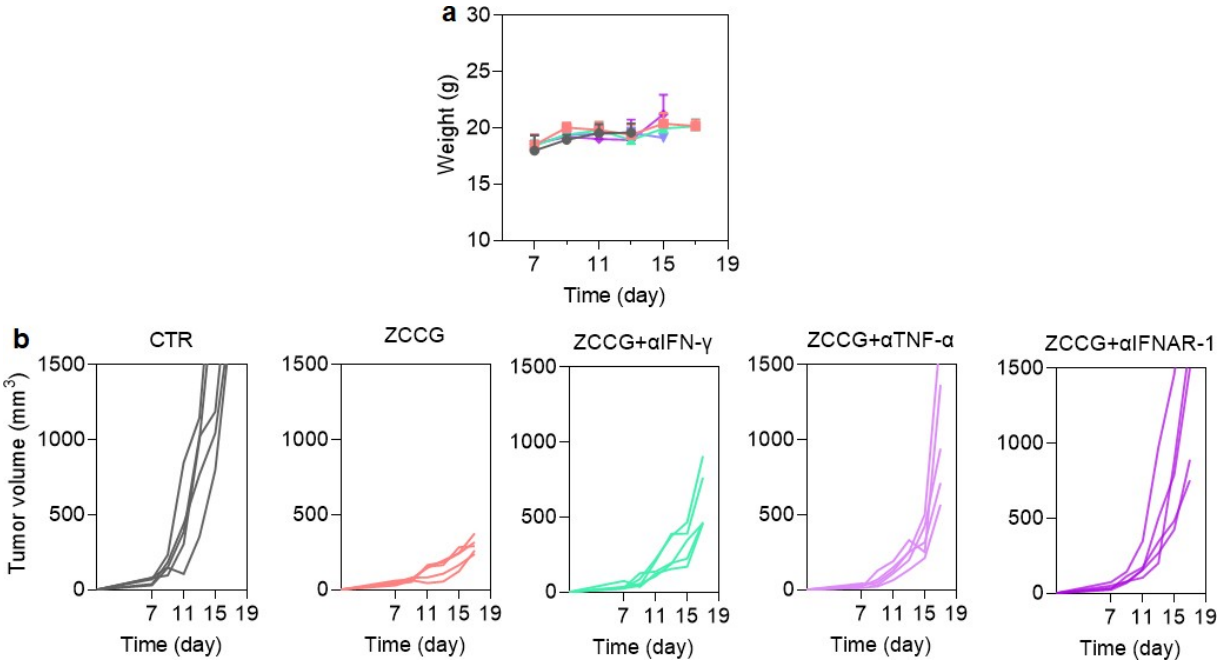

Fig. S13. a) Weight changes of mice in different groups. **b**) Individual tumor growth kinetics in different groups.

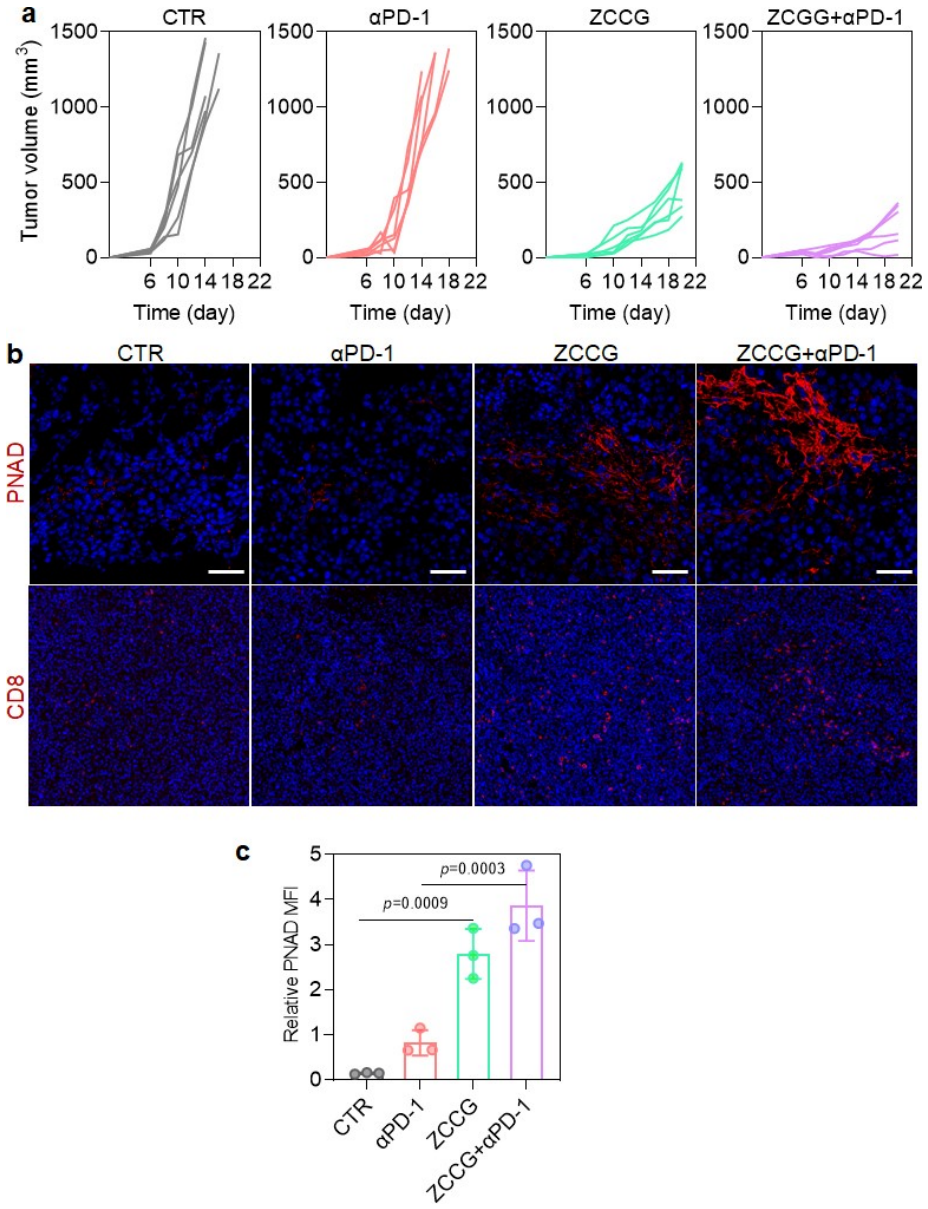

Fig. S14. a) Individual tumor growth kinetics in different groups. **b**) Immunofluorescence images of tumor sections for HVEs stained with PNAD (red). Scale bar: 50 μm. **c**) The corresponding quantification of fluorescence intensity using ImageJ software from four randomly selected images.

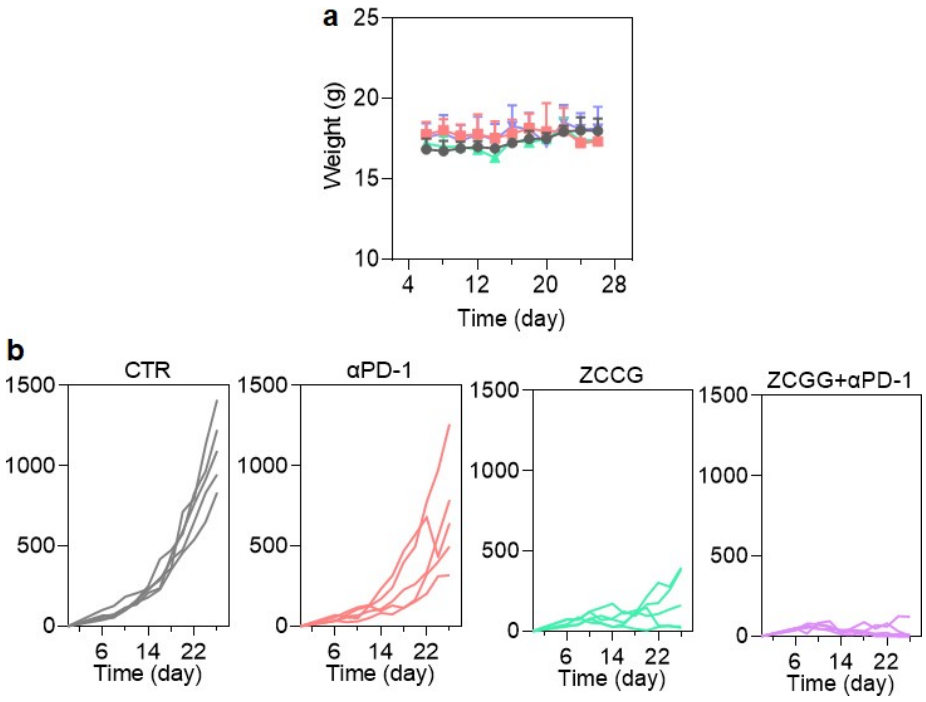

Fig. S15. a) Weight changes of mice in different groups. **b)** Individual tumor growth kinetics in different groups.

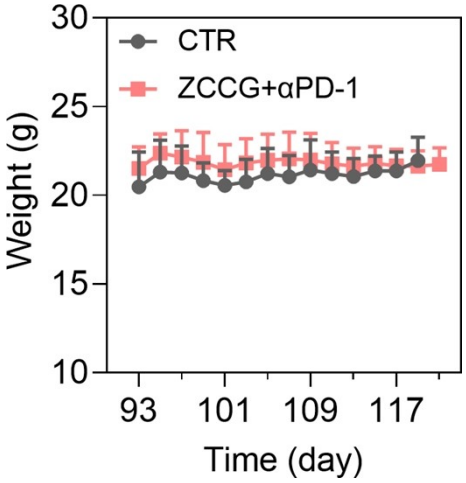

Fig. S16. Weight changes of mice in different groups.

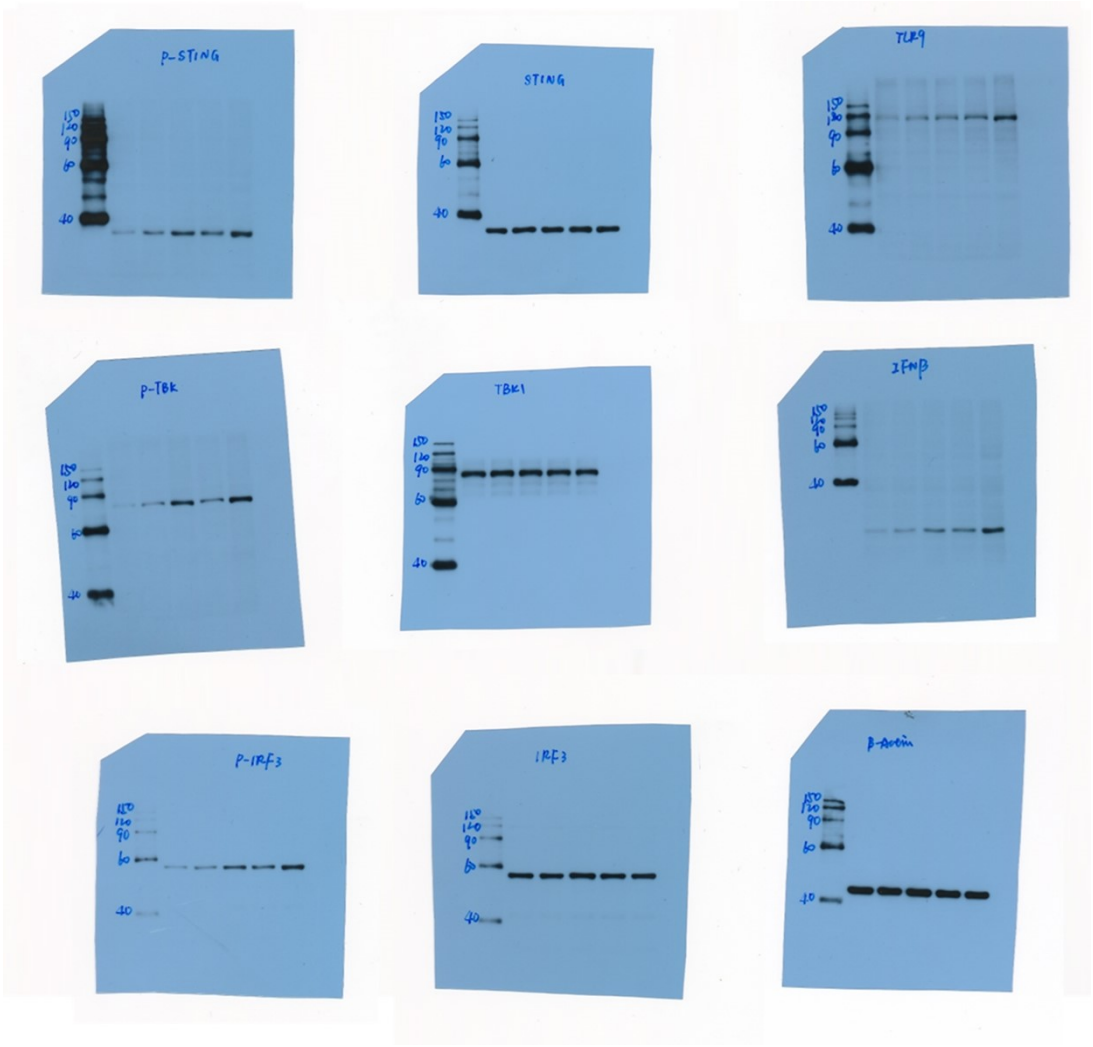

Fig. S17. The uncropped Western blot for Figure 3e.

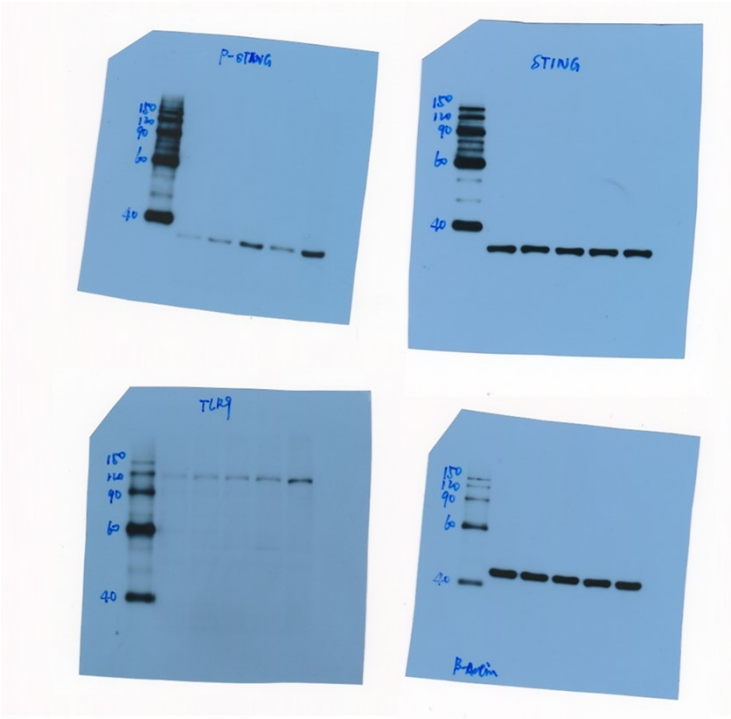

Fig. S18. The uncropped Western blot for Figure 4g.